Relationship between Radical Intensity and Cytotoxic Activity of Dopamine-Related Compounds

MASAMI KAWASE[1], NOBORU MOTOHASHI[2], TERUO KURIHARA[3], MASAHIRO INAGAKI[4], KAZUE SATOH[5] and HIROSHI SAKAGAMI[6]

[1]Faculty of Pharmaceutical Sciences and [3]Department of Chemistry, Faculty of Science, Josai University, Saitama;
[2]Department of Medicinal Chemistry, Meiji College of Pharmacy, Tokyo;
[4]Department of Chemistry, College of Arts and Sciences and
[5]Analysis Center, School of Pharmaceutical Sciences, Showa University, Tokyo;
[6]Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan

Abstract. *Millimolar concentrations of dopamine (DA), norepinephrine (NE), and 3,4-dihydroxyphenylacetic acid (DOPAC) were cytotoxic to human promyelocytic leukemic HL-60 cells. However, their metabolites (3,4-dihydroxymandelic acid (DOMA), 3-methoxytyramine (MT), normetanephrine (NMN)) and six synthetic derivatives (which have two OCH_3 groups replacing two OH groups on catechol backbone) displayed much lower cytotoxic activity. Three active compounds, but not other less potent compounds, produced radicals under alkaline conditions. All active compounds significantly enhanced the decay of ascorbic acid endogenously present in rat brain homogenate, whereas all synthetic derivatives were inactive. Ascorbic acid induced apoptotic cell death in HL-60 cells and the apoptosis induction was significantly reduced by simultaneous addition of (DA). The cytotoxic activity of (DA) was also neutralized by ascorbic acid. These data suggest the possible interaction between (DA) and ascorbic acid.*

Dopamines such as DOPA (3-hydroxytyrosine), dopamine [DA, **1**], norepinephrine (noradrenalin, NE [**2**]) and epinephrine (adrenalin) have shown antitumor activity against murine sarcoma 180 tumor (1). Sarcoma 180 tumor cells exposed to different concentrations of dopamine [**1**] showed significantly reduced ^3H thymidine incorporation into the tumor cells (2). A neurotoxic 6-hydroxydopamine retarded the growth of C1300 neuroblastoma and the A10 breast adenocarcinoma (3). NE [**2**] increased the blood supply to a rat fibrosarcoma and, when combined with methotrexate, caused a reduction in tumor size (4). DA [**1**] stimulated adenylate cyclase activity in malignant neuroblastoma cells (5). DA [**1**] possesed antitumor activity against B16 melanoma, C1300 neuroblastoma, and L1210 and P388 lymphocytic leukemias (6-8). Complex formation with ascorbic acid enhanced the cytotoxic effect of 6-hydroxydopamine, suggesting the oxygen radical generation by 6-hydroxydopamine (9-11). Third, DA [**1**] is one of a neurotransmitter and its level in the brain significantly declined in nerve diseases, such as Parkinsonism. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) acted as a free radical scavenger in murine neuroblastoma cells by reacting directly with both the 6-hydroxydopamine radical and, in the presence of iron, its oxidation product, the hydroxy radical (12). However, the mechanism by which DA induces cytotoxicity has not yet been elucidated. We have recently found that ascorbate- (13), gallate- (14) and benzo[*a*]phenothiazine-related compounds (15,16), which produced radicals, induced apoptosis or differentiation, whereas those, which did not produce radicals, were rather inactive. This suggests the importance of radical production for expression of biological activity. In extension of these studies, we investigated here the relationship between radical intensity and cytotoxic activity of authentic and synthetic DA analogs, using ESR spectroscopy.

Abbreviations: Dopamine hydrochloride [DA, **1**]; norepinephrine [NE, **2**]; 3,4-dihydroxyphenylacetic acid [DOPAC, **3**]; 3,4-dihydroxymandelic acid [DOMA, **4**]; 3-methoxytyramine [MT, **5**]; normetanephrine [NMN, **6**]; *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-*N*-methanesulfonylalanine (KD1, [**7**]); *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylalanine (KD2, [**8**]); N-(2-phenylethyl)-N-trifluoromethanesulfonylglycine (KD3, [**9**]); *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylglycine (KD4, [**10**]); *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylvaline (KD5, [**11**]); *N*-[2-(3,4-dimethoxyphenyl)ethyl]-2-phenyl-*N*-trifluoromethanesulfonyl (KD6, [**12**]); 2-(3,4-dimethoxyphenyl)ethylamine oxalate (KD7, [**13**])

Correspondence to: Dr. Masami Kawase. Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0290, Japan. Tel: (+)81-492-71-7680 (Ex.413); Fax: (+)81-492-71-7984.

Key Words: Dopamine analogs, ascorbic acid, structure-activity relationship, cytotoxicity, radical intensity, π-spin density.

Materials and Methods

Chemicals. The following chemicals were obtained from the indicated companies. DA hydrochloride [**1**], 3-methoxytyramine [MT, **5**], normetanephrine [NMN, **6**] (Sigma, U.S.A.); norepinephrine [NE, **2**], 3,4-dihydroxyphenylacetic acid [DOPAC, **3**], 3,4-dihydroxymandelic acid [DOMA, **4**] (Aldrich); RPMI1640 medium (GIBCo, Grand Island, N.Y.); ascorbic acid (Tokyo Kasei, Tokyo); sodium 5,6-benzylidene-l-ascorbate (SBA) (supplied by Dr. M. Kochi); fetal bovine serum (FBS) (JRH Biosc).

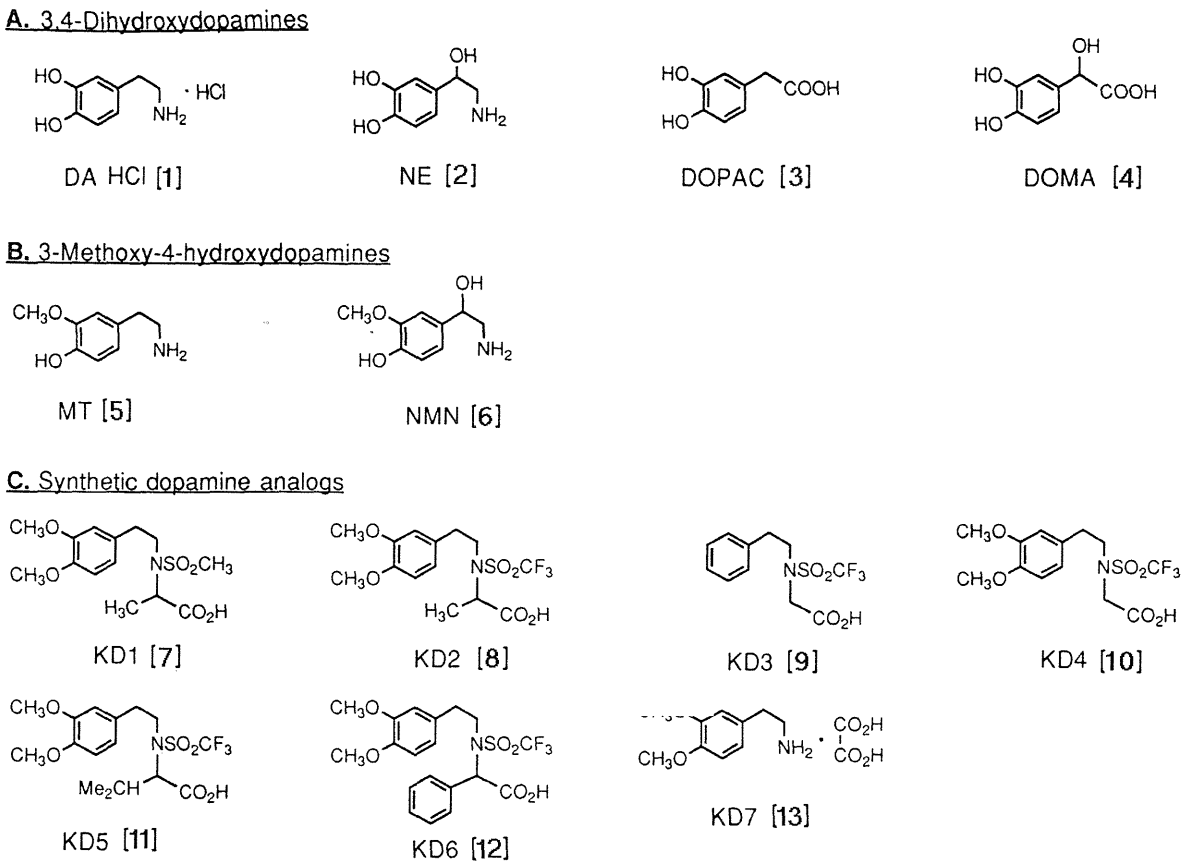

Figure 1. *The structure of 3,4-dihydroxydopamines [**1-4**], 3-methoxy-4-hydroxydopamines [**5,6**], and synthetic DA analogs [**7-13**].*

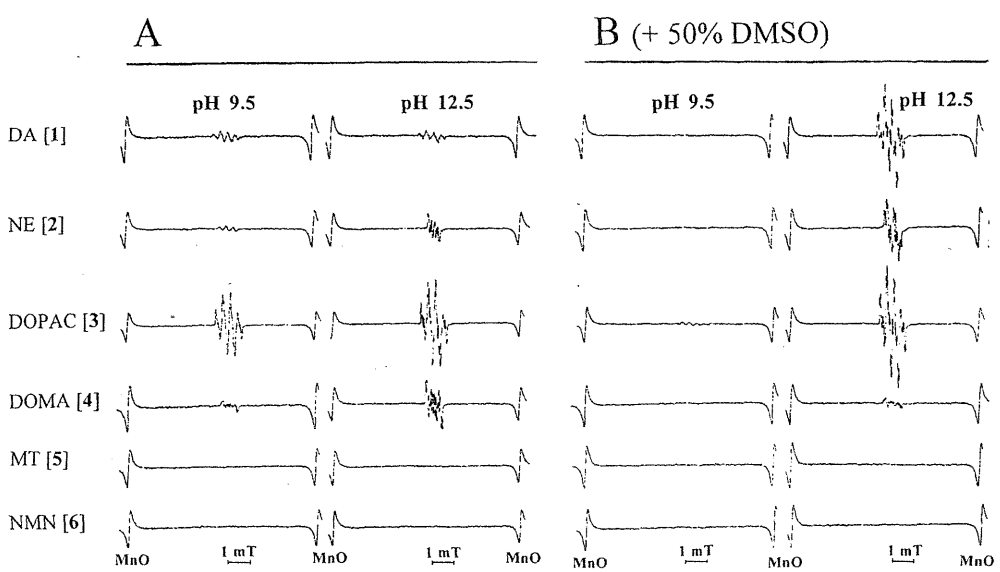

Figure 2. *ESR spectra of DA-related compounds (5 mM) measured at pH9.5 or 12.5 in the absence (**A**) or presence of 50%DMSO (**B**).*

The following seven DAs were newly synthesized as recently published (17). *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methanesulfonylalanine (KD1, [**7**], m.w. 331.11, oil), *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylalanine (KD2, [**8**], m.w. 385.36, mp115-116°C), *N*-(2-phenylethyl)-*N*-trifluoromethanesulfonylglycine (KD3, [**9**], m.w. 311.28, mp149-151°C), *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylglycine (KD4, [**10**], m.w. 371.33, mp112-113°C), *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-trifluoromethanesulfonylvaline (KD5, [**11**], m.w. 413.11, oil), *N*-[2-(3,4-dimethoxyphenyl)ethyl]-2-phenyl-*N*-trifluoromethanesulfonyglycine (KD6, [**12**], m.w. 447.10, oil) and 2-(3,4-dimethoxyphenyl)ethylamine oxalate (KD7, [**13**], m.w. 271.27, mp 181-183°C (EtOH). Anal.Calcd. for $C_{12}H_{17}NO_6$: C, 53.13; H, 6.32; N, 5.10. Found C, 53.02; H, 6.20; N, 4.93.). The structures of these compounds are shown in Figure 1.

Cell culture. Human promyelocytic leukemic HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FBS (18).

Cytotoxic activity. HL-60 cells (1 x 10^6/mL) were incubated for 24 hours with various concentrations of test samples in the cultured medium, and the viable cell number was determined by trypan blue dye exclusion (18). The 50% cytotoxic concentration (CC_{50}) of each compound was determined by dose-response curve.

Assay for radical intensity. Radical intensity of test samples was determined at 25°C exactly 1 minute after mixing samples in phosphate-buffered saline (pH7.4) 0.1M Tris-HCl (pH9.5) or 0.1M KOH (pH12.5) with ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 2-10 x 100; time constant, 0.1 second; scanning time, 4 minutes. Radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (13-16).

Preparation of homogenate. Male Wistar rats (7 weeks old) (Sankyo Labo. Tokyo) were decapitated. The whole brain was excised and, after removal of the cerebellum, was homogenized by a Teflon-glass homogenizer in 4 volumes of ice-cold Krebs Ringer solution (pH7.4) saturated with 95% oxygen.

HPLC determination of total ascorbic acid. 50 μL tissue homogenate was mixed with 450 μL 5% metaphosphoric acid containing 20 μM deferoxamine, and centrifuged at 4°C for 10 minutes at 3000 g. 150 μL of the supernatant was mixed with 50 μL dithiothreitol (50 mM) and 50 μL K_2HPO_4 (40 mM) (pH 8.6). The mixture was reacted for 20 minutes at room temperature in the dark, and added 50 μL of 50% metaphosphoric acid. After centrifugation at 3,000 *g* for 10 minutes, the supernatant was filtered through a cellulose nitrate membrane (pore size: 0.22 μm; Millipore, Bedford, MA, U.S.A.). The filtrate was immediately used to determine the total ascorbic acid. Ascorbate was analysed by HPLC system with electrochemical detection (ECD) according to the method of Iriyama with some modifications (19). The mobile phase consists of 0.2M KH_2PO_4-H_3PO_4 (pH 3) containing 50 μM EDTA. The solution was filtered through a cellulose nitrate membrane (pore size: 0.45 μm; Millipore) and degassed before use. A flow rate of 1.0 mL/min was maintained with a dual piston pump (CCPT, Tosoh, Tokyo). A 4.6 x 150-mm reverse-phase column (Wakosil-II, SC18-100; Wako Pure Chem. Ind. Ltd., Osaka) was used for separation. The applied potential on the amperometric detector (ECD-100, Eicom, Kyoto) was +650 mV.

Calculation. π-Spin density (ρ^π) of DAs [**1-13**] was calculated by unrestricted Hatree-Fock/Parametric method 3 (UHF/PM3 method) (20). The geometries of the cation radical species were optimized with respect to all geometrical parameters using Broyden-Fletcher-Goldfarb-shanno algorithm incorporated in the program. For these calculations, the FACOM M330 computer in the Josai University Information Sciences Center was used. π-Spin density (ρ^π) was defined as the α-spin density minus the ß-spin density.

Table I. *Cytotoxic activity and radical intensity of DA-related compounds [1-13].*

Compound (No.)	Cytotoxic activity (CC_{50} mM)[a]	Radical intensity (pH 12.5)[b]	Rat brain homogenate	
			Decrease of ascorbate concentration (%)[c]	Decrease of ascorbate radical intensity (%)[d]
Control	-	-	24.6	43.4
DA [**1**]	0.11	2.13	86.4	90.2
NE [**2**]	0.23	1.28	66.4	N.D.[e]
DOPAC [**3**]	0.79	3.27	61.9	N.D.
DOMA [**4**]	>10	0.26	47.2	N.D.
MT [**5**]	6.4	<0.01	49.0	N.D.
NMN [**6**]	7.6	<0.01	33.0	N.D.
KD1 [**7**]	>10	<0.01	28.7	37.1
KD2 [**8**]	3.6	<0.01	26.3	37.9
KD3 [**9**]	2.4	<0.01	32.1	34.0
KD4 [**10**]	6.2	<0.01	27.8	28.0
KD5 [**11**]	9.6	<0.01	22.4	15.6
KD6 [**12**]	0.43	<0.01	25.5	37.6
KD7 [**13**]	2.0	<0.01	29.7	33.3

a) HL-60 cells (1 x 10^6/mL) were incubated for 20 hours with various concentrations of each compound, and viable cell number was determined by trypan blue dye exclusion.
b) The radial intensity of the hihgest peak was determined by ESR spectroscopy in 50% DMSO, pH 12.5 (data from Figure 2B).
c) Determined by the difference between 0 and 60-minutes incubation (data from Figure 3).
d) Determined by the difference between 10- and 60-minutes incubation (data from Figure 4).
e) N.D.: not determined.

Results

Cytotoxicity. Millimolar concentrations of catecholamines (DA [**1**], NE [**2**]) and DOPAC [**3**] were cytotoxic to HL-60 cells. Among these compounds, the cytotoxic activity of DA [**1**] was the greatest (CC_{50} = 0.11 mM), followed by NE [**2**] (CC_{50} = 0.23 mM) and DOPAC [**3**] (CC_{50} = 0.79 mM) (Table I).

However, the cytotoxic activity of their metabolites (DOMA [**4**], MT [**5**], NMN [**6**] (CC_{50} = >10, 6.4, 7.6 mM, respectively) and six synthetic derivatives (KD1-KD5 [**7-11**] and KD7 [**13**]), which have two methoxy groups (OCH_3) replacing two hydroxyl groups (OH) or none of these functional groups in catechol backbone (CC_{50} = >10, 3.6, 2.4, 6.2, 9.6, 2.0 mM, respectively) were much less (Table I).

Radical generation. ESR spectroscopy demonstrated that DA [**1**], NE [**2**], DOPAC [**3**] and DOMA [**4**] produced radicals at

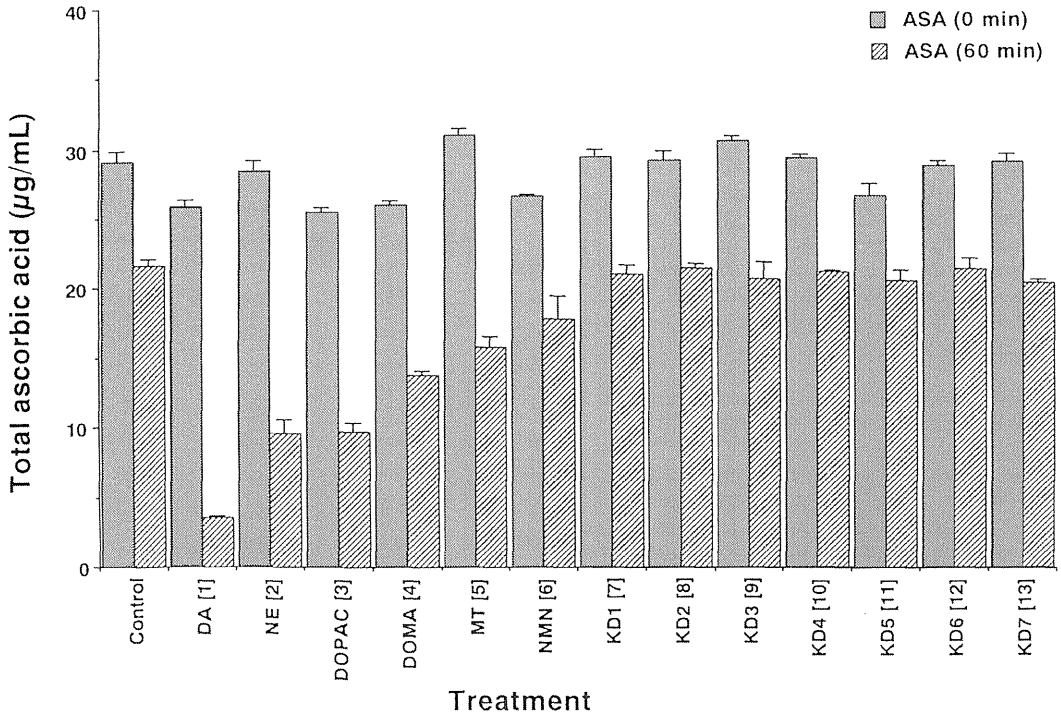

Figure 3. *Effect of DA-related compounds on the concentrations of ascorbic acid endogenously present in rat brain homogenate. Rat brain homogenate was mixed with 0.1 mM each test compound, and incubated at 37°C for 0 or 60 minutes. The remaining ascorbic acid concentration was determined by HPLC.*

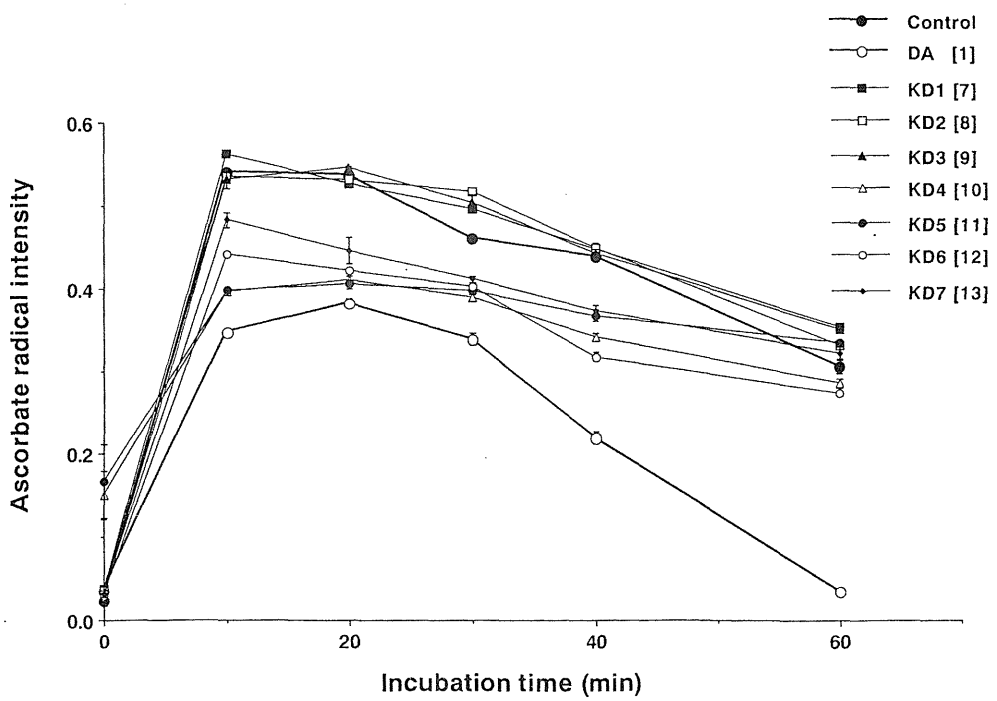

Figure 4. *Effect of DA-related compounds on the radical intensity of ascorbic acid endogenously present in rat brain homogenate. Rat brain homogenate was incubated for the indicated times at 37°C without (●) or with 0.1 mM DA[**1**] or synthetic DAs KD1-KD7 [**7-13**], and the radical intensity was determined by ESR spectroscopy.*

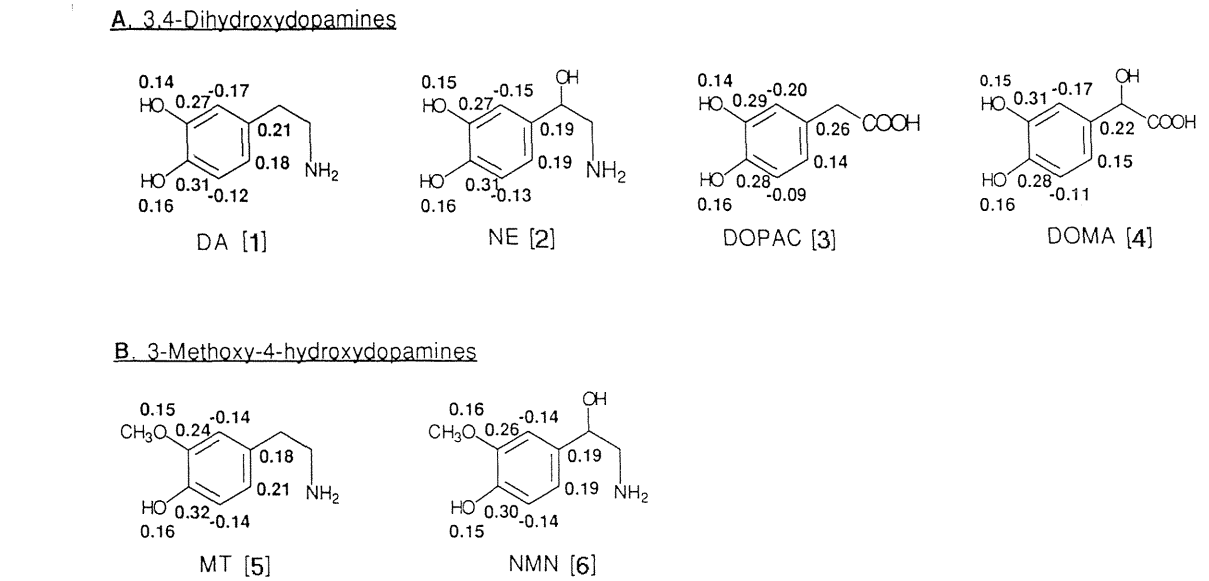

Figure 5. *π-Spin density of DA-related compounds [1-6] by UHF/PM3 method.*

pH9.5, and their radical intensity was significantly increased when the pH was elevated up to 12.5 (Figure 2A). In 50% DMSO solution, these compounds produced only minor radical intensity at pH9.5, but their radical intensity became much larger at pH12.5 (Figure 2B). It should be noted that DOMA [**4**], which did not show any significant cytotoxic activity (Table I), produced only trace amounts of radical intensity at pH12.5 (Figure 2B).

On the other hand, MT [**5**], NMN [**6**] (Figures 2A and 2B) and seven synthetic compounds KD1-7 [**7-13**] (Table I) produced no detectable radical at pH9.5-12.5.

Effect on ascorbic acid metabolism. Rat brain homogenate contained 145 μg ascorbic acid/g wet tissue. The addition of DA [**1**], NE [**2**], DOPAC [**3**] or DOMA [**4**] significantly enhanced the decay of ascorbic acid (Figure 3). MT [**5**] and NMN [**6**] showed only marginal effects. Synthetic compounds KD1-7 [**7-13**] were essentially inactive (Figure 3).

When the rat brain homogenate was incubated in Krebs Ringer solution (pH7.4), the radical intensity of ascorbic acid endogenously present in the homogenate was significantly enhanced within 10 minutes and gradually declined thereafter (Figure 4). The addition of DA [**1**] to the homogenate significantly accelerated the decay of the ascorbate radical intensity, possibly due to the loss of intact ascorbate molecule. On the other hand, the addition of any synthetic compounds (KD1-7) [**7-13**], did not significantly change the turnover pattern of ascorbate radical intensity (Figure 4).

Effect on π-spin density. Figure 5 shows that oxygen or carbon atom in the active compounds (DA [**1**], NE [**2**], DOPAC [**3**] and DOMA [**4**]) or inactive compounds (MT [**5**] and NMN [**6**]) showed similar value of π-spin density, calculated in gas phase. This indicates that their calculated values of π-spin density do not correlate with the radical intensity or cytotoxic activity of each dopamine.

Interaction between DA and ascorbate. Figure 6A shows that both DA and ascorbic acid dose-dependently reduced the viable cell number of HL-60 cells. The CC_{50} of these compounds was 0.11 mM (for DA [**1**]) and 0.5 mM (for ascorbic acid), respectively. However, combination of these two compounds neutralized their cytotoxic activity with each other, suggesting the interaction between DA and ascorbic acid (Figure 6). We also found a similar interaction between DA [**1**] and sodium 5,6-benzylidene-l-ascorbate (SBA) (Figure 6B), which showed potent tumor degeneration activity against human inoperable tumors and rat chemically-induced hepatocellular carcinoma (21).

Discussion

The present study demonstrated that a) compounds which have two *ortho* phenolic OH groups in the catechol backbone (DA [**1**], NE [**2**] and DOPAC [**3**]) were cytotoxic, and b) those which have one (MT [**5**], NMN [**6**]) or no OH group in the catechol backbone (KD1-KD5 [**7-11**], KD7 [**13**]) displayed much lower cytotoxic activity (Table I). This suggests the importance of catechol backbone for cytotoxicity induction. We found that catecholamines, which produced the radicals, were cytotoxic, whereas their derivatives, which did not produce radicals, were not cytotoxic except for KD6 [**12**] (Table I). This finding suggests that radical production might initiate the signalling pathway leading to cell death.

However, KD6 [**12**], which has no OH group but two OCH_3 group in the catechol backbone, was highly cytotoxic

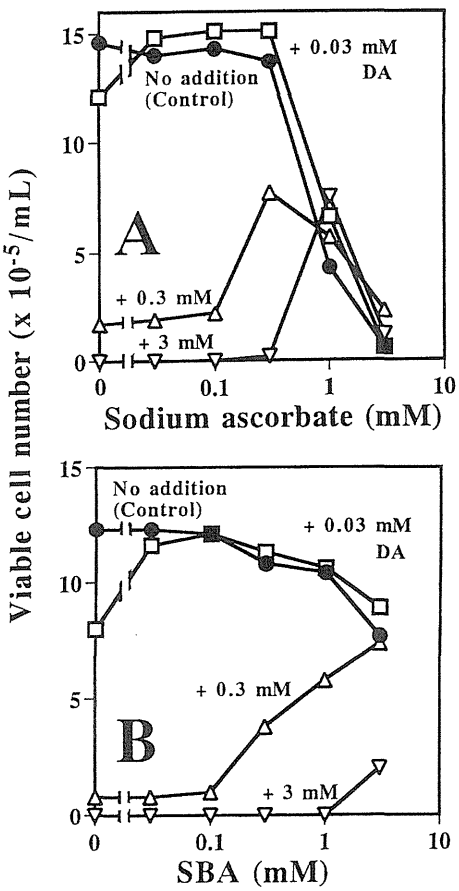

Figure 6. *Effect of sodium ascorbate or SBA on DA [1]-induced cytotoxicity in HL-60 cells.*
HL-60 cells were incubated for 24 hours with the indicated concentrations of sodium ascorbate (A) or sodium 5,6-benzylidene-l-ascorbate (SBA) (B) in the presence of 0 (●: control), 0.03 (□), 0.3 (△) or 3 (▽) mM DA [1] and the viable cell number was then determined by trypan blue dye exclusion.

(CC_{50} = 0.43 mM), even though this compound did not produce radicals (Table I). This might be due to the phenylglycine site, which is present only in the KD6 [**12**] molecule. It is predictable that radical spin density on benzyl carbon of a center of phenylglycine site might have an important role on cytotoxic activity. This indicates that catecholamines and KD6 [**12**] induce cytotoxicity by different mechanisms. Thus, the introduction of two *ortho* phenolic OH group to KD6 [**12**] might further enhance its cytotoxic activity.

The present study also demonstrated that DA [**1**] enhanced the decay of ascorbic acid in rat brain homogenate (Figure 3). The possibility should be investigated that DA [**1**] might enhance the degradation of ascorbic acid or simply make a complex with ascorbic acid (9-11), which elutes at different retention times on HPLC separation. We found that DA [**1**] and ascorbic acid interacted with each other, thus reducing their individual cytotoxic activity. This suggests that ascorbic acid might affect the DA metabolism, and dDA-mediated nerve diseases such as Parkinsonism. Further study of the changes in the redox potential after treatment with DA [**1**] and ascorbic acid is underway in our laboratory.

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